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(54) Title: NEW PRIMERS AND PROBES FOR THE DETECTION OF PARVOVIRUS B19

(57) Abstract: The present invention relates to new primers and probes for the detection of parvovirus B19 as well as to kits containing them. Further, the invention relates to methods wherein the new primers and probes can be used, in particular homogeneous polymerase chain reaction methods. Further, the invention relates to uses of the new primers and probes.

## New primers and probes for the detection of parvovirus B19

### Field of the invention

The present invention relates to new primers and probes for the detection of parvovirus B19 as well as to kits containing them. Further, the invention relates to methods wherein the new primers and probes can be used, in particular  
5 homogeneous polymerase chain reaction methods. Further, the invention relates to uses of the new primers and probes.

### Background of the invention

Parvovirus B19 infection is a common childhood illness which usually runs a mild course in immunocompetent individuals, producing a characteristic rash known as  
10 erythema infectiosum or fifth disease (Anderson M.J. et al., Lancet 1 (1983) 1378). Infection may be complicated by severe arthralgia or a transient aplastic crisis in individuals suffering from chronic hemolytic disease (J. R. et al., Lancet 1 (1981) 664-665). Congenital anemia and vasculitis have also been described (Cohen B., BMJ 311 (1995) 1549-1552). More recently the virus has been associated with  
15 hepatitis and myocarditis (Yoto Y. et al., Lancet 347 (1996) 868-9; Enders G. et al., Clin Infect Dis 26 (1998) 355-358; Dux S. et al., Dtsch Med Wochenschr 127 (2002) 1584-1588). Following maternal infection in pregnancy the virus may be transmitted to the fetus, causing hydrops, spontaneous abortion or intrauterine death (Enders E: Infections of the fetus and the neonate other than rubella. Topley  
20 & Wilson's Microbiology and Microbial Infections. Edited by Collier L. London, Edward Arnold, 1998, pp. 873-915). Besides transmission via the respiratory route, parvovirus B19 infection may also occur through contaminated blood and blood products (Brown K. E., Baillieres Best Pract Res Clin Haematol 13 (2000) 245-259). The latter has been recognized by the US Food and Drug Administration, resulting  
25 in a proposal for parvovirus B19 nucleic acid testing (NAT) to be regarded as in-process testing rather than donor screening.

In the field of molecular diagnostics, the detection of target nucleic acids with the polymerase chain reaction (PCR) plays an important role. The routine screening of blood banks for the presence of Human Immunodeficiency Virus (HIV), or  
30 Hepatitis-B (HBV) or C Virus (HCV) is an example for the large-scale application of PCR-based diagnostics. Automated systems for PCR-based analysis often make

use of real-time detection of product amplification during the PCR process. Key to such methods is the use of modified oligonucleotides carrying reporter groups or labels. The detection of DNA amplification products generated by a PCR process can, on the one hand, be accomplished in separate working steps. These may 5 involve the characterisation of amplified fragments with respect to their electrophoretic mobility and/or the analysis of denatured amplification products attached to a solid support using a hybridisation probe.

On the other hand, the detection of DNA amplification products can be done in a so-called "homogeneous" assay system. A "homogeneous" assay system comprises 10 reporter molecules or labels which generate a signal while the target sequence is amplified. An example for a "homogeneous" assay system is the TaqMan® system that has been detailed in US 5,210,015, US 5,804,375 and US 5,487,972. Briefly, the method is based on a double-labelled probe and the 5'-3' exonuclease activity of Taq DNA polymerase. The probe is complementary to the target sequence to be 15 amplified by the PCR process and is located between the two PCR primers during each polymerisation cycle step. The probe has two fluorescent labels attached to it. One is a reporter dye, such as 6-carboxyfluorescein (FAM), which has its emission spectra quenched by energy transfer due to the spatial proximity of a second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). In the course of each 20 amplification cycle, the Taq DNA polymerase in the process of elongating a primed DNA strand displaces and degrades the annealed probe, the latter due to the intrinsic 5'-3' exonuclease activity of the polymerase. The mechanism also frees the reporter dye from the quenching activity of TAMRA. As a consequence, the fluorescent activity increases with an increase in cleavage of the probe, which is 25 proportional to the amount of PCR product formed. Accordingly, amplified target sequence is measured detecting the intensity of released fluorescence label. Another example for "homogeneous" assay systems are provided by the formats used in the LightCycler® instrument (see e.g. US 6,174,670), some of them sometimes called "Kissing probe" formats. Again, the principle is based on two interacting dyes 30 which, however, are characterised in that the emission wavelength of a donor-dye excites an acceptor-dye by fluorescence resonance energy transfer. The COBAS® AmpliPrep instrument (Roche Diagnostics GmbH, D-68305 Mannheim, Germany) was recently introduced to expand automation by isolating target sequences using biotinylated sequence-specific capture probes along with streptavidin-coated 35 magnetic particles (Jungkind D., J Clin Virol 20 (2001) 1-6; Stelzl E. et al., J Clin Microbiol 40 (2002) 1447-1450). It has lately been joined by an additional versatile

tool, the Total Nucleic Acid Isolation (TNAI) Kit (Roche Diagnostics). This laboratory-use reagent allows the generic, not sequence-specific isolation of all nucleic acids from plasma and serum on the COBAS® AmpliPrep instrument based essentially on the method developed by Boom R. et al., J Clin Microbiol 28 (1990) 5 495-503.

- Assay systems for parvovirus B19 are disclosed in Japanese unexamined patent publication No. 147986/1995 or by Schorling, S. et al., J. Mol. Diagn. 6 (2004), 37-41. Primers for the detection of parvovirus B19 from the VP1 or VP2 region are disclosed in US 6,274,307. The cloning of VP1 and VP2 are disclosed in JP 10 04088985. A probe for parvovirus RA-1 is disclosed in EP 238 893 and probes for parvovirus B19 in WO 01/06019. The NS1 gene and probes thereto are disclosed in EP 783 580. PCR based detection of Parvovirus B19 is described in RU2146372. A nucleic acid sequence of a specific erythrovirus is described in WO 99/28439. WO 03/002753 describes a diagnostic assay for parvovirus B19. WO 02/00924 15 describes parvoviral phospholipase A2. A method for determining large quantities of parvovirus B19 is described in US 6,183,999. Testing for parvovirus B19 is disclosed in WO 01/14593. DNA control constructs are disclosed in WO 02/096925. Parvovirus-like particles are described in WO 91/04330. JP 11221099 describes PCR 20 amplification of parvovirus B19. A method for treating parvovirus B19 infections is disclosed in US 6,268,349. Autonomous parvovirus B19 gene delivery vehicles are described in US 5,585,254 and corresponding US patents and WO 00/24917.

The sequence of parvovirus B19 is described in Shade, R. O. et al., J Virol 58 (1986) 921-936 and the analysis of the genome is described by Cotmore S. F. et al., J. Virol. 60 (1986) 548-557 and Ozawa K. et al., J. Virol. 62 (1988) 2508-2511. A detection 25 method for parvovirus B19 is described in Sato K. et al., J Clin Microbiol 38 (2000) 1241-1243, Cubie H. A. et al., Mol Cell Probes 9 (1995), 59-66, Jordan J. A. et al., Mol. Diagn. 1 (1996), 321-328, Vassias I. et al., J. Virol. Meth. 44 (1993) 329-338, and Carriere C. et al., J Virol Methods 44 (1993), 221-234, and Holloway B. et al., Nucleic Acids Res 21 (1993), 3905-3906. The VP1 region is analysed by Dorsch S. et 30 al., J. Gen. Virol. (2001) 82, 191-199 and Takahashi N. et al., FEBS Lett. 450 (1999) 289-293. The NS1 region is analysed by Hicks K. E. et al., Arch Virol. 141 (1996), 1319-1327. The sequence variability among different parvovirus B19 isolates is described in Hemauer A. et al., J. Gen. Virol. (1996) 77, 1781-1785, Umene K. and Nunoue T., J. Gen. Virol. 76 (1995) 2645-2651, Erdman D. D. et al., J Gen Virol 77

(1996), 2767-2774 and Astell et al., J. Gen. Virol. 68 (1987) 885-893, and Turton J. et al., Epidemiol Infect 105 (1990) 197-201.

WO99/28439 describes certain primers and probes suitable for the detection of erythrovirus V9 and B19. US 6,395,472 discloses nucleic oligomer primers or 5 probes useful for detection of TT virus. Schmidt et al. (Vox Sanguinis (2001) 81, 228-235) disclose the detection of parvovirus B19 in plasma pools and plasma derivatives.

#### Summary of the invention

Because of the clinical significance of parvovirus B19, there is a need for further 10 primers and probes that can detect the virus in biological samples.

Therefore, the invention provides a method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of

- 15     (a) providing a sample suspected to contain the target nucleic acid  
         (b) providing a pair of primers comprising a first and a second primer whereby  
            the first primer consists of at least contiguous 12 nucleotides of a nucleic acid  
            sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby  
            the second primer consists of at least contiguous 12 nucleotides of a nucleic  
            acid sequence selected from the complementary sequence of the nucleic acid  
            sequences SEQ ID NO: 3 or 4,  
         (c) amplifying the target nucleic acid,  
         (d) detecting the amplified target nucleic acid of step (c).
- 20     The invention further provides a method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of
  - (a) providing a sample suspected to contain the target nucleic acid,  
         (b) providing a pair of primers comprising a first and a second primer,  
         (c) amplifying the target nucleic acid,  
         (d) contacting the sample with a probe under conditions for binding the probe to  
            the target nucleic acid,
- 25
- 30

- (e) detecting the binding product between the target nucleic acid and the probe as an indication of the presence of the target nucleic acid
- characterized in that
- 5 the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4, and/or
- 10 the probe consists of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 5 or a complementary sequence thereof.
- The invention further provides an oligonucleotide whereby the nucleic acid sequence of the oligonucleotide is selected from the nucleic acid sequences SEQ ID NO: 12 to 15, from the nucleic acid sequences 10 or 11 or a complementary
- 15 15 sequence thereof or from the complementary sequence of the nucleic acid sequences 16 or 17.
- In another embodiment of the invention, a pair of primers is provided comprising a first and a second primer whereby the nucleic acid sequence of the first primer is selected from the nucleic acid sequences SEQ ID NO: 12 to 15 and whereby the
- 20 20 nucleic acid sequence of the second primer is selected from the complementary sequence of the nucleic acid sequences 16 or 17.
- The oligonucleotides or the pair of primers according to the invention can be used in a hybridisation reaction with a complementary nucleic acid. In another embodiment of the invention, the oligonucleotides or the pair of primers according
- 25 25 to the invention can be used as a primer, probe or capture probe.
- The invention further provides a kit comprising a template dependent DNA polymerase, nucleotides and an oligonucleotide or a pair of primers according to the invention.

**Detailed description of the invention**

- Conventional techniques of molecular biology and nucleic acid chemistry, which are within the skill of the art, are explained in the literature. See, for example, Sambrook J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor 5 Laboratory Press, Cold Spring Harbor, New York, 1989, Gait, M.J., ed., 1984; Nucleic Acid Hybridization, Hames, B.D., and Higgins, S.J., eds., 1984; and a series, Methods in Enzymology, Academic Press, Inc., all of which are incorporated herein by reference. All patents, patent applications, and publications mentioned herein, both *supra* and *infra*, are incorporated herein by reference.
- 10 Before nucleic acids may be analysed in one of the above-mentioned assays, they have to be isolated or purified from biological samples containing complex mixtures of different components. Often, for the first steps, processes are used which allow the enrichment of the nucleic acids. To release the contents of cells or viral particles, they may be treated with enzymes or with chemicals to dissolve, 15 degrade or denature the cellular walls or the viral particles. This process is commonly referred to as lysis. The resulting solution containing such lysed material is referred to as lysate. A problem often encountered during the lysis is that other enzymes degrading the component of interest, e.g. desoxyribonucleases or ribonucleases degrading nucleic acids, come into contact with the component of 20 interest during lysis. These degrading enzymes may also be present outside the cells or may have been spatially separated in different cellular compartments before the lysis and come now into contact with the component of interest. Other components released during this process may be e.g. endotoxins belonging to the family of lipopolysaccharides which are toxic to cells and can cause problems for products 25 intended to be used in human or animal therapy.

There are a variety of means to tackle this problem mentioned-above. It is common to use chaotropic agents as e.g. guanidinium thiocyanate or anionic, cationic, zwitterionic or non-ionic detergents when nucleic acids are intended to be set free. It is also an advantage to use proteases which rapidly degrade these enzymes or 30 unwanted proteins. However, this may produce another problem as the said substances or enzymes can interfere with reagents or components in subsequent steps.

Enzymes which can be advantageously used in such lysis or sample preparation processes mentioned-above are enzymes which cleave the amide linkages in protein substrates and which are classified as proteases, or (interchangeably) peptidases (see Walsh, 1979, Enzymatic Reaction Mechanisms. W. H. Freeman and Company, San Francisco, Chapter 3). Proteases which have been used in the prior art are e.g. alkaline proteases (WO 98/04730) or acid proteases (US 5,386,024). The protease which is widely used in the prior art for sample preparation for the isolation of nucleic acids is proteinase K from *Tritirachium album* (see e.g. Sambrook J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 10 Cold Spring Harbor, New York, 1989) which is active around neutral pH and belongs to a family of proteases known to the person skilled in the art as subtilisins.

In the next steps of the sample preparation which follow on the lysis step, the component of interest is further enriched. If the non-proteinaceous components of interest are e.g. nucleic acids, they are normally extracted from the complex lysis 15 mixtures before they are used in a probe-based assay.

There are several methods for the extraction of nucleic acids:

- sequence-dependent or biospecific methods as e.g.:
  - affinity chromatography
  - hybridisation to immobilised probes
- 20 - sequence-independent or physico-chemical methods as e.g.:
  - liquid-liquid extraction with e.g. phenol-chloroform
  - precipitation with e.g. pure ethanol
  - extraction with filter paper
  - extraction with micelle-forming agents as cetyl-trimethyl-ammonium-bromide
  - 25 • binding to immobilised, intercalating dyes, e.g. acridine derivatives
  - adsorption to silica gel or diatomic earths
  - adsorption to magnetic glass particles (MGP) or organo silane particles under chaotropic conditions
- 30 Particularly interesting for extraction purposes is the adsorption of nucleic acids to a glass surface although other surfaces are possible. Many procedures for isolating nucleic acids from their natural environment have been proposed in recent years by

the use of their binding behavior to glass surfaces. If unmodified nucleic acids are the target, a direct binding of the nucleic acids to a material with a silica surface is preferred because among other reasons the nucleic acids do not have to be modified and even native nucleic acids can be bound. These processes are described in detail  
5 by various documents. In Vogelstein B. et al., Proc. Natl. Acad. USA 76 (1979) 615-9, for instance, a procedure for binding nucleic acids from agarose gels in the presence of sodium iodide to ground flint glass is proposed. The purification of plasmid DNA from bacteria on glass dust in the presence of sodium perchlorate is described in Marko M. A. et al., Anal. Biochem. 121 (1982) 382-387. In  
10 DE-A 37 34 442, the isolation of single-stranded M13 phage DNA on glass fiber filters by precipitating phage particles using acetic acid and lysis of the phage particles with perchlorate is described. The nucleic acids bound to the glass fiber filters are washed and then eluted with a methanol-containing Tris/EDTA buffer. A similar procedure for purifying DNA from lambda phages is described in Jakobi R.  
15 et al., Anal. Biochem. 175 (1988) 196-201. The procedure entails the selective binding of nucleic acids to glass surfaces in chaotropic salt solutions and separating the nucleic acids from contaminants such as agarose, proteins or cell residue. To separate the glass particles from the contaminants, the particles may be either centrifuged or fluids are drawn through glass fiber filters. This is a limiting step,  
20 however, that prevents the procedure from being used to process large quantities of samples. The use of magnetic particles to immobilize nucleic acids after precipitation by adding salt and ethanol is more advantageous and described e.g. in Alderton R. P. et al., S., Anal. Biochem. 201 (1992) 166-169 and PCT GB 91/00212. In this procedure, the nucleic acids are agglutinated along with the magnetic  
25 particles. The agglutinate is separated from the original solvent by applying a magnetic field and performing a wash step. After one wash step, the nucleic acids are dissolved in a Tris buffer. This procedure has a disadvantage, however, in that the precipitation is not selective for nucleic acids. Rather, a variety of solid and dissolved substances are agglutinated as well. As a result, this procedure can not be  
30 used to remove significant quantities of any inhibitors of specific enzymatic reactions that may be present. Magnetic, porous glass is also available on the market that contains magnetic particles in a porous, particular glass matrix and is covered with a layer containing streptavidin. This product can be used to isolate biological materials, e.g., proteins or nucleic acids, if they are modified in a complex  
35 preparation step so that they bind covalently to biotin. Magnetizable particular adsorbents proved to be very efficient and suitable for automatic sample preparation. Ferrimagnetic and ferromagnetic as well as superparamagnetic

pigments are used for this purpose. The most preferred MGPs and methods using magnetic glass particles are those described in WO 01/37291 which is incorporated by reference herein.

After the purification or isolation of the nucleic acids including the target nucleic acid from their natural surroundings, the target nucleic acid may be detected. Therefore, in one embodiment of the invention, a method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample is provided comprising the steps of

- (a) providing a sample suspected to contain the target nucleic acid
- 10 (b) providing a pair of primers comprising a first and a second primer whereby the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4,
- 15 (c) amplifying the target nucleic acid,
- (d) detecting the amplified target nucleic acid of step (c).

Preferably, the method does not comprise the step of providing the sample suspected to contain the target nucleic acid. Therefore, in one embodiment of the invention, a method is provided for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of

- (a) amplifying the target nucleic acid in a sample suspected to contain the target nucleic acid in the presence of a pair of primers comprising a first and a second primer whereby the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4, and
- 25 (b) detecting the amplified target nucleic acid of step (a).

Preferably, the first primer consists of at least 12 contiguous nucleotides of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 6 or 7 and

wherein the second primer consists of at least 12 contiguous nucleotides of the complementary sequence of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 8 or 9. Preferably, the primer consists of 15 or 18 contiguous nucleotides of the said nucleic acid sequences or a complementary 5 thereof. More preferably, the first primer has a nucleic acid sequence selected from the group of nucleic acid sequences SEQ ID NO: 12 to 15 and wherein the second primer has a nucleic acid sequence selected from the complementary sequence of the group of nucleic acid sequences SEQ ID NO: 16 to 17. It is preferred that the primers are chosen to amplify the amplicon according to SEQ ID NO: 1.

10

As is known in the art, a "nucleoside" is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines.

"Nucleotides" are "nucleosides" that further include a phosphate group covalently 15 linked to the sugar portion of the nucleoside. For those "nucleosides" that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. A "nucleotide" is the "monomeric unit" of an "oligonucleotide", more generally denoted herein as an "oligomeric compound", or a "polynucleotide", more generally denoted as a "polymeric compound". Another 20 general expression therefor is desoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

According to the invention, an "oligomeric compound" is a compound consisting of "monomeric units" which may be "nucleotides" alone or "non-natural compounds" (see below), more specifically "modified nucleotides" (or "nucleotide 25 analogs") or "non-nucleotide compounds", alone or combinations thereof. "Oligonucleotides" and "modified oligonucleotides" (or "oligonucleotide analogs") are subgroups of "oligomeric compounds" in the context of the invention.

In the context of this invention, the term "oligonucleotide" refers to "polynucleotides" formed from a plurality of "nucleotides" as the "monomeric 30 unit", i.e. an "oligonucleotide" belongs to a specific subgroup of a "oligomeric compound" or "polymeric compound" of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) with "monomeric units". The phosphate groups are commonly referred to as forming the internucleoside backbone of the

"oligonucleotide". The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

"Oligonucleotides" and "modified oligonucleotides" (see below) according to the invention may be synthesized as principally described in the art and known to the expert in the field. Methods for preparing oligomeric compounds of specific sequences are known in the art, and include, for example, cloning and restriction of appropriate sequences and direct chemical synthesis. Chemical synthesis methods may include, for example, the phosphotriester method described by Narang S. A. et al., *Methods in Enzymology* 68 (1979) 90-98, the phosphodiester method disclosed 5 by Brown E. L. et al., *Methods in Enzymology* 68 (1979) 109-151, the phosphoramidite method disclosed in Beaucage et al., *Tetrahedron Letters* 22 (1981) 1859, the H-phosphonate method disclosed in Garegg et al., *Chem. Scr.* 25 (1985) 280-282 and the solid support method disclosed in US 4,458,066.

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As said above, a "nucleic acid" as well as the "target nucleic acid" is a polymeric 15 compound of "nucleotides" as known to the expert skilled in the art. It is used herein to denote a "nucleic acid" in a sample which should be analyzed, i.e. the presence, non-presence or amount thereof in a sample should be determined. Therefore, in other words the "nucleic acid" is the target and can therefore be also denoted as "target nucleic acid". For example, if it has to be determined whether 20 blood contains parvovirus B19, the "target nucleic acid" is the nucleic acid of the Parvovirus B19. The term "complementary (nucleic acid) sequence of a nucleic acid sequence" as used herein means that the complementary (nucleic acid) sequence referred to is exactly the (inverse) complement of the nucleic acid sequence.

The term "primer" is used herein as known to the expert skilled in the art and refers 25 to "oligomeric compounds" primarily to "oligonucleotides" but also to "modified oligonucleotides" that are able to "prime" DNA synthesis by a template-dependent DNA polymerase, i.e. the 3'-end of the e.g. oligonucleotide provides a free 3'-OH group whereto further "nucleotides" may be attached by a template-dependent DNA polymerase establishing 3' to 5' phosphodiester linkage whereby 30 desoxynucleoside triphosphates are used and whereby pyrophosphate is released. Therefore, there is - except for the intended function - no fundamental difference between a "primer", an "oligonucleotide" or a "probe" according to the invention.

As used herein, the singular forms "a," "an" and "the" include both singular and plural references unless the content clearly dictates otherwise.

- As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are  
5 additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a  
10 method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, etc.
- 15 The amplification is performed preferably with the polymerase chain reaction which specifically amplifies target nucleic acids to detectable amounts. Other possible amplification reactions are the Ligase Chain Reaction (LCR; Wu D. Y. and Wallace R. B., Genomics 4 (1989) 560-69; and Barany F., Proc. Natl. Acad. Sci. USA 88 (1991)189-193); Polymerase Ligase Chain Reaction (Barany F., PCR Methods  
20 and Appl. 1 (1991) 5-16); Gap-LCR (WO 90/01069); Repair Chain Reaction (EP 0439182 A2), 3SR (Kwoh D. Y. et al., Proc. Natl. Acad. Sci. USA 86 (1989) 1173-1177; Guatelli J.C., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 1874-1878; WO 92/08808), and NASBA (US 5,130,238). Further, there are strand displacement amplification (SDA), transcription mediated amplification (TMA), and Q $\beta$ -  
25 amplification (for a review see e.g. Whelen A. C. and Persing D. H., Annu. Rev. Microbiol. 50 (1996) 349-373; Abramson R. D. and Myers T. W., Curr Opin Biotechnol 4 (1993) 41-47).

- Suitable DNA detection methods are known to the expert in the field and are  
30 described in standard textbooks as Sambrook J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989 and Ausubel F. et al.: Current Protocols in Molecular Biology 1987, J. Wiley and Sons, NY. There may be also further purification steps before the DNA detection step is carried out as e.g. a precipitation step. The detection methods may  
35 include but are not limited to the binding or intercalating of specific dyes as

ethidiumbromide which intercalates into the double-stranded DNA and changes its fluorescence thereafter. The purified DNA may also be separated by electrophoretic methods optionally after a restriction digest and visualized thereafter. There are also probe-based assays which exploit the oligonucleotide hybridisation to specific sequences and subsequent detection of the hybrid. It is also possible to sequence the DNA after further steps known to the expert in the field. The preferred template-dependent DNA polymerase is Taq polymerase.

A preferred embodiment of the invention are therefore the above-described purification method followed by a determination or detection step or purification methods followed by an amplification and determination or detection step.

In another embodiment of the invention, a method is provided for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of

- 15 (a) providing a sample suspected to contain the target nucleic acid,
- (b) providing a pair of primers comprising a first and a second primer,
- (c) amplifying the target nucleic acid,
- (d) contacting the sample with a probe under conditions for binding the probe to the target nucleic acid,
- 20 (e) detecting the binding product between the target nucleic acid and the probe as an indication of the presence of the target nucleic acid

characterized in that

the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4, and/or

the probe consists of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 5 or a complementary sequence thereof.

Preferably, the method does not comprise the step of providing the sample suspected to contain the target nucleic acid. Therefore, in another embodiment of

the invention, a method is provided for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of

- (a) amplifying the target nucleic acid in a sample suspected to contain the target nucleic acid in the presence of a pair of primers comprising a first and a second primer,
- (b) contacting the sample of step a) with a probe under conditions for binding the probe to the target nucleic acid,
- (c) detecting the binding product between the target nucleic acid and the probe as an indication of the presence of the target nucleic acid

characterized in that

the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4, and/or

the probe consists of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 5 or a complementary sequence thereof.

The term „probe“ refers to synthetically or biologically produced nucleic acids (DNA or RNA) which, by design or selection, contain specific nucleotide sequences that allow them to hybridize under defined predetermined stringencies specifically (i.e., preferentially) to “target nucleic acids”. A „probe“ can be identified as a capture probe“ meaning that it “captures” the target nucleic acid so that it can be separated from undesirable materials which might obscure its detection. Once separation is accomplished, detection of the captured “target nucleic acid” can be achieved using a suitable procedure. „Capture probes“ are often already attached to a solid phase.

In the method according to the invention, the probe consists preferably of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 10 or a complementary sequence thereof. Preferably, the probe consist of at least 15 or 18 contiguous nucleotides of the nucleic acid sequences of the invention. More

preferably, the probe has the nucleic acid sequence SEQ ID NO: 11 or a complementary sequence thereof.

In a preferred embodiment of the method according to the invention, the first 5 primer consists of at least 12 contiguous nucleotides of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 6 or 7 and wherein the second primer consists of at least 12 contiguous nucleotides of the complementary sequence of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 8 or 9. In a more preferred embodiment of the invention, the first primer 10 has a nucleic acid sequence selected from the group of nucleic acid sequences SEQ ID NO: 12 to 15 and wherein the second primer has a nucleic acid sequence selected from the complementary sequence of the group of nucleic acid sequences SEQ ID NO: 16 to 17. Preferably, the primers are chosen to allow of the amplification of the amplicon with the nucleic acid sequence according to SEQ ID 15 NO: 1.

The method according to the invention may be performed in a format for the use in the LightCycler® instrument which is described in US 6,174,670. This format comprises amplification and detection whereby the latter uses the detection of the fluorescence for the detection of the binding product between a pair of probes and 20 the target nucleic acid. These formats apply the fluorescent resonance energy transfer technology (see, for example, US Patent Nos. 4,996,143, 5,565,322, 5,849,489, and 6,162,603) and are based on the fact that when a donor and a corresponding acceptor fluorescent label are positioned within a certain distance of each other, energy transfer takes place between the two fluorescent labels that can 25 be visualized or otherwise detected and/or quantitated. As used herein, two probes, each containing a fluorescent label, whereby at least one thereof is an oligonucleotide according to the invention, can hybridize to an amplification product at particular positions determined by the complementarity of the probes to the target nucleic acid. The fluorescent label according to the invention of the 30 oligonucleotide according to the invention may be a donor or acceptor fluorescent label. Upon hybridization of the probes to the amplification product at the appropriate positions, a FRET signal is generated. Fluorescent analysis can be carried out using, for example, a photon counting epifluorescent microscope system (containing the appropriate dichroic mirror and filters for monitoring 35 fluorescent emission at the particular range), a photon counting photomultiplier system, or a fluorometer. Excitation to initiate energy transfer can be carried out

- with an argon ion laser, a high intensity mercury (Hg) arc lamp, a fiber optic light source, or other high intensity light source appropriately filtered for excitation in the desired range. As used herein with respect to donor and corresponding acceptor fluorescent labels, "corresponding" refers to an acceptor fluorescent label having an
- 5 excitation spectrum that overlaps the emission spectrum of the donor fluorescent label. Accordingly, efficient non-radiative energy transfer can be produced there between. The preferred fluorescent label is fluorescein as the donor fluorescent label, whereby the acceptor fluorescent label is rhodamine, however, preferred is a cyanine dye, preferably Cy5 as described in US 6,174,670.
- 10 "Labels", often referred to as "reporter groups", are generally groups that make a nucleic acid, in particular the "oligomeric compound" or the "modified oligonucleotide" according to the invention, as well as any nucleic acids bound thereto distinguishable from the remainder of the liquid, i.e. the sample (nucleic acids having attached a "label" can also be termed labeled nucleic acid binding
- 15 compounds, labeled probes or just probes). Preferred labels according to the invention are fluorescent labels, which are e.g. fluorescent dyes as a fluorescein dye, a rhodamine dye, a cyanine dye, and a coumarin dye.

As used herein, "fluorescence resonance energy transfer relationship" and similar terms refer to adjacent hybridization of an "oligonucleotide" labeled with a "donor

20 fluorescent label" and another "oligomeric compound" labeled with an "acceptor fluorescent label" to a "target nucleic acid" such that the "donor fluorescent label" can transfer resonance energy to the "acceptor fluorescent label" such that the "acceptor fluorescent label" produces a measurable fluorescence emission. If the "donor fluorescent label" and "acceptor fluorescent label" are spaced apart by too

25 great a distance, then the "donor fluorescent label" cannot transfer resonance energy to the "acceptor fluorescent label" such that the "acceptor fluorescent label" emits measurable fluorescence, and hence the "donor fluorescent label" and "acceptor fluorescent label" are not in resonance energy transfer relationship.

In a preferred embodiment of the invention, the probe carries a label. Preferably, an

30 additional probe carrying a label is contacted with the sample in step d) so that a pair of probes consisting of a first and a second probe is contacted with the sample in step d).

In a preferred embodiment of the invention, a method according to the invention is provided wherein said amplifying step c) in the method according to the invention comprises contacting the sample with the said pair of primers to produce an amplification product if the target nucleic acid is present in said sample, wherein

5   said hybridizing step d) comprises contacting said sample with the pair of probes, wherein the members of said pair of probes hybridize to said amplification product within no more than five nucleotides of each other, wherein the first probe of said pair of probes is labeled with a donor fluorescent label and wherein the second probe of said pair of probes is labeled with a corresponding acceptor fluorescent

10   label; and detecting the binding product between the target nucleic acid and the pair of probes in step e) by detecting the presence or absence of fluorescence resonance energy transfer between said donor fluorescent label of said first probe and said acceptor fluorescent label of said second probe, wherein the presence of fluorescence resonance energy transfer is indicative of the presence of the target

15   nucleic acid in the sample, and wherein the absence of fluorescence resonance energy transfer is indicative of the absence of the target nucleic acid in the sample.

Therefore, in an embodiment of the invention, a method for detecting the presence or absence of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample is provided, comprising the steps of performing at least

20   one cycling step, wherein a cycling step comprises an amplifying step and a hybridizing step, wherein said amplifying step comprises contacting said sample with primers, whereby a primer is an oligonucleotide according to the invention, to produce an amplification product if target nucleic acid is present in said sample, wherein said hybridizing step comprises contacting said sample with the pair of

25   probes, wherein the members of said pair of probes hybridize to said amplification product within no more than five nucleotides of each other, wherein a first probe of said pair of probes is labeled with a donor fluorescent label and wherein a second probe of said pair of probes is labeled with a corresponding acceptor fluorescent label, wherein a probe may be an oligonucleotide according to the invention; and

30   detecting the presence or absence of fluorescence resonance energy transfer between said donor fluorescent label of said first probe and said acceptor fluorescent label of said second probe, wherein the presence of FRET is indicative of the presence of the target nucleic acid in the sample, and wherein the absence of FRET is indicative of the absence of the target nucleic acid in the sample.

In another preferred embodiment of the invention, a method for detecting a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample is provided, comprising the steps of amplifying the nucleic acid by polymerase chain reaction in the presence of two nucleic acid probes, whereby a probe may be an oligonucleotide according to the invention, that hybridize to adjacent regions of the target nucleic acid, one of said probes being labeled with an acceptor fluorescent label and the other probe labeled with a donor fluorescent label of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target nucleic acid, the donor and acceptor fluorescent labels are within 25 nucleotides of one another, said polymerase chain reaction comprising the steps of adding a thermostable polymerase, nucleotides and primers, whereby a primer may be an oligonucleotide according to the invention, for the target nucleic acid to the sample and thermally cycling the sample between at least a denaturation temperature and an elongation temperature; exciting the biological sample with light at a wavelength absorbed by the donor fluorescent label and detecting fluorescent emission from the fluorescence energy transfer pair.

In another preferred embodiment of the invention, a method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in sample is provided comprising the steps of amplifying the nucleic acid by polymerase chain reaction in the presence of two nucleic acid probes, whereby a probe may be an oligonucleotide according to the invention, that hybridize to adjacent regions of the nucleic acid, one of said probes being labeled with an acceptor fluorescent label and the other probe labeled with donor fluorescent label of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target nucleic acid, the donor and acceptor fluorescent labels are within 25 nucleotides of one another, said polymerase chain reaction comprising the steps of adding a thermostable polymerase, nucleotides and primers, whereby a primer may be an oligonucleotide according to the invention, for the target nucleic acid to the sample and thermally cycling the sample between at least a denaturation temperature and an elongation temperature; exciting the sample with light at a wavelength absorbed by the donor label and monitoring temperature dependent fluorescence from the fluorescence energy transfer pair.

In a preferred embodiment of the method of the invention, the format used in the TaqMan® assay is contemplated. This format comprises amplification and detection whereby the latter uses the detection of the fluorescence for the detection

of the binding product the probe and the target nucleic acid. Therefor, the probe, which may be an oligonucleotide according to the invention, comprises a label, which is preferably a fluorescent label, preferably fluorescein. The probe may further comprise other fluorescent labels wherein the emission wavelengths of one 5 of the fluorescent labels overlaps the absorption wavelengths of another of the fluorescent labels. Preferably, the probe further comprises a second fluorescent label acting as a quenching agent, that quenches the fluorescence emission of the fluorescent label, which can be fluorescein. Preferably the quenching agent is a fluorescent rhodamine or cyanine. The quenching agent can also be a non- 10 fluorescent compound or dye as dabcyl ("Dark quencher"). The probe cannot be extended enzymatically to be used as probe in the TaqMan® format as principally set out in US 5,210,015, US 5,478,972, or US 5,804,375. Preferably, the monomeric unit at the 3'-end of the oligomeric compound is a 2',3'-dideoxynucleotide or a 3'-phosphorylated nucleotide. In consequence for the format used in the TaqMan® 15 assay, in the determination step of the method, the spatial relationship between the fluorescent label and the second label, i.e. the quenching agent, subsequent to hybridization is altered, preferably by exonuclease hydrolysis of a template-dependent DNA polymerase, preferably the Taq-Polymerase, of the probe whereby release of label occurs as a result of exonuclease hydrolysis. Therefore, in the 20 method according to the invention, the target nucleic in step c) is amplified with a template-dependent DNA polymerase. The degree of hybridization between the oligomeric compound according to the invention and the nucleic acid is determined by the quantity of label that is released from the probe subsequent to hybridization. Therefore it is a preferred embodiment of the invention, that in step 25 25 (d) the degree of hybridization is determined by the quantity of label that is released from the probe hybridized to the nucleic acid by exonuclease hydrolysis by the template-dependent DNA polymerase.

Therefore, more preferably, the probe, which may be an oligonucleotide according to the invention carries a first and a second label. In the most preferred 30 embodiment, in step (e) the degree of hybridization or the binding product of the probe and the target nucleic acid is determined by the quantity of the first or second fluorescent label that is released from the probe hybridized to the target nucleic acid by exonuclease hydrolysis by the template-dependent DNA polymerase.

35 In a very preferred embodiment of the invention related in more detail to the TaqMan® assay format, a method for the detection of a target nucleic acid

comprising the nucleic acid sequence of parvovirus B19 in a sample is provided comprising the steps of

- (a) contacting a sample comprising single-stranded nucleic acids with a primer or oligonucleotide containing a sequence complementary to a region of the target nucleic acid and a probe containing a first and second fluorescent label, and whereby said probe contains a sequence complementary to a second region of the same target nucleic acid sequence strand, but not including the nucleic acid sequence defined by the primer or oligonucleotide, to create a mixture of duplexes during hybridization conditions, wherein the duplexes 5 comprise the target nucleic acid annealed to the primer or oligonucleotide and to the probe such that the 3' end of the first primer or oligonucleotide is upstream of the 5' end of the probe,
- (b) maintaining the mixture of step (a) having a 5' to 3' nuclease activity under conditions sufficient to permit the 5' to 3' nuclease activity of the polymerase 10 to cleave the annealed probe and release labelled fragments; and
- (c) detecting and/or measuring the release of labelled fragments.

For the above-described method, the nucleic acids can be present in double-stranded or single-stranded form whereby the double-stranded nucleic acids are 20 denatured, i.e. made single-stranded, before the method is performed by heating, i.e. thermal denaturing.

In another preferred embodiment, a primer and/ or the probe may be chemically modified, i.e. the primer and/ or the probe comprise a modified nucleotide or a 25 non-nucleotide compound. The probe or the primer is then a modified oligonucleotide.

“Modified nucleotides” (or “nucleotide analogs”) differ from a natural “nucleotide” by some modification but still consist of a base, a pentofuranosyl sugar, a phosphate 30 portion, base-like, pentofuranosyl sugar-like and phosphate-like portion or combinations thereof. For example, a “label” may be attached to the base portion of a “nucleotide” whereby a “modified nucleotide” is obtained. A natural base in a “nucleotide” may also be replaced by e.g. a 7-desazapurine whereby a “modified nucleotide” is obtained as well. The terms “modified nucleotide” or “nucleotide 35 analog” are used interchangeably in the present application. A “modified nucleoside” (or “nucleoside analog”) differs from a natural nucleoside by some

modification in the manner as outlined above for a "modified nucleotide" (or a "nucleotide analog").

A "non-nucleotide compound" is different from a natural "nucleotide" but is in the sense of this invention still capable - similar to a "nucleotide" - of being a 5 "monomeric unit" of an "oligomeric compound". Therefore, a "non-nucleotide compound" has to be capable of forming an "oligomeric compound" with "nucleotides". Even "non-nucleotide compounds" may contain a base-like, pentofuranosyl sugar-like or a phosphate-like portions, however, not all of them are present at the same time in a "non-nucleotide compound".

- 10 A "modified oligonucleotide" (or "oligonucleotide analog") belongs to another specific subgroup of the "oligomeric compounds", that possesses one or more "nucleotides", one or more "non-nucleotide compounds" or "modified nucleotides" as "monomeric units". Thus, the terms "modified oligonucleotide" (or "oligonucleotide analog") refers to structures that function in a manner 15 substantially similar to "oligonucleotides" and are used interchangeably throughout the application. From a synthetical point of view, a "modified oligonucleotide" (or a "oligonucleotide analog") can be for example made by chemical modification of "oligonucleotides" by appropriate modification of the phosphate backbone, ribose unit or the nucleotide bases (Uhlmann and Peyman, Chemical Reviews 90 (1990) 543; 20 Verma S., and Eckstein F., Annu. Rev. Biochem. 67 (1998) 99-134). Representative modifications include phosphorothioate, phosphorodithioate, methyl phosphonate, phosphotriester or phosphoramidate inter-nucleoside linkages in place of phosphodiester inter-nucleoside linkages; deaza or aza purines and pyrimidines in place of natural purine and pyrimidine bases, pyrimidine bases 25 having substituent groups at the 5 or 6 position; purine bases having altered substituent groups at the 2, 6 or 8 positions or 7 position as 7-deazapurines; sugars having substituent groups at, for example, their 2' positions, or carbocyclic or acyclic sugar analogs. Other modifications consistent with the spirit of this invention are known to those skilled in the art. Such "modified oligonucleotides" (or 30 "oligonucleotide analogs") are best described as being functionally interchangeable with, yet structurally different from, natural "oligonucleotides" (or synthetic "oligonucleotides" along natural lines). In more detail, exemplary modifications are disclosed in Verma S., and Eckstein F., Annu. Rev. Biochem. 67 (1998) 99-134 or WO 02/12263. In addition, modification can be made wherein nucleoside units are 35 joined through groups that substitute for the internucleoside phosphate or sugar

phosphate linkages. Such linkages include those disclosed in Verma S., and Eckstein F., Annu. Rev. Biochem. 67 (1998) 99-134. When other than phosphate linkages are utilized to link the nucleoside units, such structures have also been described as "oligonucleosides".

- 5 Another preferred embodiment is related to multiplex detection of various target nucleic acids, preferably different viruses. Therefore, in a preferred embodiment of the invention a method according to the invention is provided wherein other target nucleic acids are detected in the same reaction. Preferably, the other target nucleic acids comprise nucleic acid from hepatitis A virus, hepatitis B virus, hepatitis C
- 10 virus, west nile virus, the Cytomegalovirus (CMV), the human immunodeficiency virus or bacterial pathogens responsible for bacterial infections as e.g. Neisseria gonorrhoeae or chlamydial infections.

- In another embodiment of the invention, an oligonucleotide is provided whereby
- 15 15 the nucleic acid sequence of the oligonucleotide is selected from the nucleic acid sequences SEQ ID NO: 12 to 15, from the nucleic acid sequences 10 or 11 or a complementary sequence thereof or from the complementary sequence of the nucleic acid sequences 16 or 17. In a preferred embodiment of the invention, an oligonucleotide according to the invention is provided which comprises a modified
- 20 nucleotide or a non-nucleotide compound.

- In another embodiment of the invention, a pair of primers is provided comprising a first and a second primer whereby the nucleic acid sequence of the first primer is selected from the nucleic acid sequences SEQ ID NO: 12 to 15 and whereby the
- 25 25 nucleic acid sequence of the second primer is selected from the complementary sequence of the nucleic acid sequences 16 or 17.

- An oligonucleotide or a pair of primers according to the invention can be used in a hybridisation reaction with a complementary nucleic acid. In another embodiment of the invention, the oligonucleotide according to the invention is used as a primer,
- 30 probe or capture probe.

In another preferred embodiment a kit of parts is contemplated by the invention whereby the kit contains a template-dependent polymerase having 3' to 5' exonuclease activity, preferably the Taq Polymerase, nucleotides and oligonucleotides or a pair of primers according to the invention. In another

embodiment of the invention, a kit is provided comprising a template dependent DNA polymerase, nucleotides and an oligonucleotide or a pair of primers according to the invention.

Such kits known in the art further comprise plastics ware which can be used during 5 the amplification procedure as e.g. microtitre plates in the 96 or 384 well format or just ordinary reaction tubes manufactured e.g. by Eppendorf, Hamburg, Germany and all other reagents for carrying out the method according to the invention.

In another embodiment of the invention, the kit contains further reagents for isolating the nucleic acid. Therefore, the kit can additionally contain a material with 10 an affinity to nucleic acids, preferably the material with an affinity to nucleic acids comprises a material with a silica surface. Preferably, the material with a silica surface is a glass. Most preferably, the material with an affinity to nucleic acids is a composition comprising magnetic glass particles as described in WO 96/41811 or WO 01/37291. The kit can further or additionally comprise a lysis buffer containing 15 e.g. chaotropic agents, detergents or alcohols or mixtures thereof which allows the lysis of cells and separately a protease, e.g. proteinase K, for the digestions of unwanted proteins. These components of the kit according to the invention may be provided separately in tubes or storage containers. Depending on the nature of the components, these may be even provided in a single tube or storage container. The 20 kit may further or additionally comprise a washing solution which is suitable for the washing step of the magnetic glass particles when DNA or RNA is bound thereto. This washing solution may contain ethanol and/or chaotropic agents in a buffered solution or solutions with an acidic pH without ethanol and/or chaotropic agents as described above. Often the washing solution or other solutions are provided as 25 stock solutions which have to be diluted before use. The kit may further or additionally comprise an eluent or elution buffer, i.e. a solution or a buffer (e.g. 10 mM Tris, 1 mM EDTA, pH 8.0) or pure water to elute the DNA or RNA bound to the magnetic glass particles. Further, additional reagents or buffered solutions may be present which can be used for the purification process of a nucleic acid, i.e. DNA 30 or RNA.

A preferred embodiment of the present invention is to use the method or the kit of the present invention in automatable methods as e.g. described in WO 99/ 16781. Automatable method means that the steps of the method are suitable to be carried out with an apparatus or machine capable of operating with little or no external

control or influence by a human being. Automatized method means that the steps of the automatable method are carried out with an apparatus or machine capable of operating with little or no external control or influence by a human being. Only the preparation steps for the method may have to be done by hand, e.g. the storage 5 containers have to filled up and put into place, the choice of the samples has to be done by a human being and further steps known to the expert in the field, e.g. the operation of the controlling computer. The apparatus or machine may e.g. add automatically liquids, mix the samples or carry out incubation steps at specific temperatures. Typically, such a machine or apparatus is a robot controlled by a 10 computer which carries out a program in which the single steps and commands are specified. Preferred automatized methods are those which are carried out in a high-throughput format which means that the methods and the used machine or apparatus are optimized for a high-throughput of samples in a short time. In another embodiment of the invention the methods or the kits according to the 15 present invention are used in semi-automatized process which means that some reaction steps may have to be done manually. In a preferred embodiment of the invention, a suspension containing MGPs according to the present invention is taken from a storage container and partial volumes are added to different reaction vessels. Reaction vessels may be reaction tubes made from plastics eventually in 20 microtitreplate format contain 96 or 384 or more wells where a reaction can be carried out. However, these vessels may be made from other material e.g. from steel.

In preferred embodiments of the invention the kit according to the invention is used for research, bioanalytics or diagnostics. In preferred embodiments according 25 to the invention the kit according to the invention or the method according to the invention is used in a high-throughput format, i.e. in an automatized method which allows the analysis of a high number of different samples in a very short time.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in 30 the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

**Description of the Figures**

- Figure 1      Kinetic PCR growth curves for the primer combination STS12/16 along with probe STS15 basing on an eluate derived from a 1000 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable.
- 5
- Figure 2      Kinetic PCR growth curves for the primer combination STS13/16 along with probe STS15 basing on an eluate derived from a 1000 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable.
- 10
- Figure 3      Kinetic PCR growth curves for the primer combination STS14/16 along with probe STS15 basing on an eluate derived from a 1000 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable.
- 15
- Figure 4      Analysis of amplification products derived from the experiments in Example 1 by means of standard agarose gel electrophoresis (E-Gel system, Invitrogen, Carlsbad, CA, USA). (Lane 1: 100bp ladder DNA; 2: STS12/16: Water control; 3: STS12/16: 1000 IU/mL sample; 4: STS12/16: 1000 IU/mL sample; 5: STS13/16: 1000 IU/mL sample; 6: STS13/16: 1000 IU/mL sample; 7: STS14/16: 1000 IU/mL sample; 8: STS14/16: 1000 IU/mL sample; 9: empty; 10: 100bp ladder DNA)
- 20
- Figure 5      Kinetic PCR growth curves for the primer combination STS14/16 compared to the primer combination STS17/18 along with probe STS15 basing on an eluate derived from a 1000 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable.
- 25
- 30

- Figure 6 Analysis of amplification products derived from the experiments in Example 2 by means of standard agarose gel electrophoresis (B-Gel system, Invitrogen, Carlsbad, CA, USA). (Lane 1: 100bp ladder DNA; 2: 50 IU/mL sample; PCR positive; 3: 25 IU/mL sample; PCR positive; 4: 25 IU/mL sample; PCR negative; 5: 10 IU/mL sample; PCR negative; 6: 10 IU/mL sample; PCR negative; 7: 10 IU/mL sample; PCR negative; 8: 10 IU/mL sample; PCR positive; 9: Negative Control; PCR negative; 10: Negative Control; PCR negative; 11: STS17/18: Water Control; PCR negative; 12: 100bp ladder DNA)
- Figure 7 Kinetic PCR growth curves for the primer combination STS17/18 along with probe STS15 basing on an eluate derived from a 1E+04 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable.
- Figure 8 Schematic drawing of the target region in the parvovirus B19 genome.

#### Examples – Detection of parvovirus B19 by Nucleic acid testing

- General:
- Sample preparation of EDTA plasma, citrate plasma and human serum has been performed employing the Total Nucleic Acid Isolation (TNAI) Kit on the COBAS AmpliPrep Instrument (both Roche Diagnostics GmbH, D-68305 Mannheim, Germany) following the manufacturer's instructions with 200 µL specimen input volume. 50 µL of eluate were then manually transferred to specific PCR tubes (referred to as k-tubes) and mixed with 50 µL of PCR reaction mix. Amplification and detection of nucleic acids was performed by kinetic PCR on the COBAS TaqMan Analyzer (Roche Diagnostics GmbH, D-68305 Mannheim, Germany) with the following PCR profile:

Step	Type	Ramp Slope (0.1 °C /s)	Temp. (°C)	Time (sec.)	Delay (sec.)	Number of Cycles
1	Cover Heating	3	100.0			1
2	PreCycle	12	50	300		1
4	PreCycle	12	95	120		1
5	Sequence Start					
6	Denaturation	12	95.0	15		
7	Annealing	12	58	25	25	
8	Sequence End					60
9	PostCycle	12	40	120		1

**Example 1****Comparison of different Primer combinations**

A TaqMan probe (STS15) comprising a highly conserved region of the NS 1 gene was designed. The probe has the sequence SEQ ID NO: 11 5'-CCCCGGGACCAGTTCAGGAGAACAT-3' (nt 2070-2095 according to Shade R. O. et al., J. Virol. 58 (1986) 921-936). The probe has a melting temperature (Tm) of approx. 80 °C when the Nearest Neighbor Method is applied (OLIGO, Molecular Biology Insights, Inc, CO, USA). Next, primer sequences have been designed to fit the following criteria:

- Tm 59-63°C
- no or less mismatches with published Erythrovirus sequences incl. recently discovered new variants (Nguyen Q. T. et al., Virology 301 (2002) 374-80; Servant A. et al., J. Virol. 76 (2002) 9124-34)
- 15 • less false priming sites
- primer should end with A or C

The following sequences were deemed suitable and have been synthesized:

- STS12 (fwd primer): 5'-GTGGTGAAAGCTCTGAAGAA-3' (SEQ ID NO: 12)  
 20 STS13 (fwd primer): 5'-GAAACCCCGCGCTCTA-3' (SEQ ID NO: 13)  
 STS14 (fwd primer): 5'-AAACCCCGCGCTCTAGTA-3' (SEQ ID NO: 14)

STS17 (fwd primer): 5'-GAAACCCCGCGCTAGTAC-3' (SEQ ID NO: 15)

STS16 (rev primer): 5'-TTCCATCCATTATACCAAGC-3' (SEQ ID NO: 16)

STS18 (rev primer): 5'-CCCAACTAACAGITCACGAA-3' (SEQ ID NO: 17)

- 5 The performance of the primer combinations STS12/16, STS13/16, STS14/16, and STS17/18 was evaluated with the probe STS15 and a PCR reaction mix consisting of 50 mM Tricine pH 8.3, 100 mM potassium acetate, 3 mM manganese acetate, 4 % glycerol, 300  $\mu$ M dATP, 300  $\mu$ M dCTP, 300  $\mu$ M dGTP, 50  $\mu$ M dTTP, 500  $\mu$ M dUTP, 10 U uracil-N-glycosylase, 40 U Z05 polymerase, 200 nM NTQ21-46A-
- 10 Aptamer, 400 nM each primer and 100 nM probe. Samples consisting of 500 and 1000 IU/mL parvovirus B19 DNA were processed on COBAS AmpliPrep instrument using the TNAI Kit. Afterwards, 50  $\mu$ L of the corresponding eluate were mixed with a 2 fold reaction mix in a k-tube and placed in the COBAS TaqMan Analyzer for Amplification/Detection.
- 15 Figures 1 through 3 show the kinetic PCR growth curves for the primer combinations STS12/16, STS13/16 and STS14/16 basing on an eluate derived from a 1000 IU/mL sample. The vertical lines indicate when the growth curves cross the threshold, in other words, when an unambiguous and specific signal is initially detectable. The crossing points of these vertical lines with the X axis are determined
- 20 as cycle threshold (ct)-values and are directly proportional to the input concentration of the target sequence. The lower a ct-value the higher the initial input of target copies. From the here presented primer combinations STS14/16 appears to be superior as judged by the lower ct-values.

- 25 Figure 4 shows analysis of the corresponding amplicons by means of agarose gel electrophoresis. It demonstrates that the PCR reactions with the given primer combinations do exhibit none or less cross reactivity or amplification of unspecific sequences.

- 30 A second reverse primer, STS18, has been evaluated along with the forward primer STS17 and the primer combination has been compared with STS14/16 with a reaction mix and PCR protocol as described above. As samples served eluates derived from replicated extractions of a 500 IU/mL specimen. Figure 5 depicts a comparison of the two primer combinations. As evident from here, STS17/18 has been chosen for further experiments since it comprises the lowest ct-values along with less deviation on ct-value basis.

**Example 2****Analytical Sensitivity with primers STS17, STS18 and probe STS15**

- For determination of sensitivity, dilution series of the World Health Organisation Standard for Parvovirus B19 DNA (National Institute for Biological Standards and Control [NIBSC] 1<sup>st</sup> International Standard 2000 Parvovirus B19 DNA 500000 IU/mL; Code 99/800) from 1000-10 IU/mL in EDTA plasma were processed in twelve replicates using the extraction method described above with 200 µl specimen input volume. Fifty microliters of eluate were manually transferred to K-tubes, mixed with 50 µl activated MMx and subsequently taken for PCR (for details, see Example 1). According to the Probit analysis algorithm the sensitivity was found to be 26 IU/mL at 95 % hitrate. Table 1 summarizes the results of the study and depicts the hitrates at the corresponding input concentration. Figure 6 shows agarose gel electrophoresis analysis of selected amplification products.

**Table 1:**

NR	Concentration IU/mL	Number of Trials	Number of Positives	Hit rate
1	500	12	12	100%
2	400	12	12	100%
3	300	11	11	100%
4	200	12	12	100%
5	100	11	11	100%
6	75	12	12	100%
7	50	11	11	100%
8	25	11	10	91%
9	10	12	9	75%

15

**Example 3****Precision testing with different primer combinations**

- Precision with the primer combination STS17/18 has been evaluated in comparison to the combination STS14/16 along with probe STS15. The Pelispy Parvo-B19-DNA run control at 1E4 IU/mL (VQC Laboratory, Alkmaar, NL) has been

- 30 -

extracted in 46 replicates on two different days following the procedure described above. The eluates have been analysed by kinetic PCR as described in Example 1. The overall CV on basis of the ct-values was found to be 2.18% in the case of the primer combination STS17/18 whereas the combination STS14/16 comprises a CV  
5 of 4.89% (Table 2).

Table 2:

	ct-values	
	Primer combination STS14/STS16	Primer combination STS17/STS18
Day 1	39,8	35,1
	40,0	33,5
	41,6	34,3
	40,6	35,7
	41,2	36,9
	40,0	35,0
	40,6	34,3
	42,0	34,8
	40,0	35,1
	41,0	35,5
	41,0	35,4
	41,3	35,4
	40,9	34,8
	42,5	35,4
	41,3	33,2
	43,4	34,4
	40,9	35,1
	42,4	36,0
	43,2	36,7
	42,1	35,8
	44,4	35,5
	41,8	35,3
	40,9	36,7
Day 2	39,8	35,5
	40,2	35,1
	36,4	34,5
	36,3	35,7
	37,8	35,8

- 31 -

	ND	34,5
	38,4	35,2
	40,2	35,4
	39,5	34,7
	37,5	35,2
	40,5	35,5
	36,2	35,6
	38,4	35,3
	39,5	35,9
	37,2	35,6
	38,6	37,4
	37,7	35,2
	41,7	35,5
	44,3	35,8
	38,6	36,4
	40,1	35,5
	39,0	34,8
	41,4	35,0
<b>mean</b>	40,3	35,3
<b>SD</b>	1,968	0,770
<b>CV</b>	4,89	2,18

**Example 4****Specificity testing with different primer combinations**

Specificity has been evaluated with different primer combinations and probe STS15 located within the NS1 gene of the Parvovirus B19 genome. The general procedure (reaction mix, PCR protocol) was adapted from Example 1. Fresh routine blood donations were supplied by the Blood Donation Center of the German Red Cross, Munich, BRD, and have been extracted as described above. The following Table 3 depicts the results from this analysis and demonstrates a specificity of 100% for all investigated combinations.

Table 3:

int. Nr.	BRK-Plasma Nr.	PCR Test with STS13/ STS16	PCR Test with STS14/ STS16	PCR Test with STS17/ STS18
301	0926091	negative	ND	ND
302	0926033	negative	negative	negative
303	0926094	negative	negative	negative
304	0926037	negative	negative	negative
305	0926086	negative	negative	negative
306	0926038	ND	ND	negative
307	0926092	ND	ND	negative
308	0926049	ND	ND	negative
309	0926061	ND	ND	negative
310	0926068	ND	ND	negative
311	0926042	ND	ND	negative
312	0926076	ND	negative	negative
313	0926043	negative	negative	negative
314	0926073	negative	negative	negative
315	0926046	negative	negative	negative
316	0926072	negative	negative	negative
317	0926067	negative	negative	negative
318	0926050	negative	negative	negative
319	0926090	ND	ND	negative
320	0926079	negative	negative	negative
321	0926053	negative	negative	negative
322	0926051	negative	negative	negative
323	0926063	negative	negative	negative
324	0926088	negative	negative	negative
325	0926071	negative	negative	ND
326	0926034	negative	negative	negative
327	0926039	negative	negative	negative
328	0926087	negative	negative	negative
329	0926095	negative	negative	negative
330	0926036	negative	negative	negative
331	0926093	negative	negative	negative
332	0926064	negative	negative	negative
333	0926065	negative	negative	negative
334	0926085	negative	negative	negative
335	0926029	negative	negative	negative
336	0926032	negative	negative	negative

int. Nr.	BRK-Plasma Nr.	PCR Test with STS13/ STS16	PCR Test with STS14/ STS16	PCR Test with STS17/ STS18
337	0926082	ND	negative	negative
338	0926098	ND	negative	negative
339	0926047	negative	negative	negative
340	0926097	negative	negative	negative
341	0941982	negative	negative	negative
342	0926069	negative	negative	negative
343	0926041	negative	negative	negative
344	0941983	negative	negative	negative
345	0926066	negative	negative	negative
346	0926035	negative	negative	negative
347	0926089	negative	ND	negative
348	0926045	negative	ND	negative
349	0926084	ND	ND	ND
350	0941971	negative	negative	negative
351	0926054	negative	negative	negative
352	0941962	negative	negative	negative
353	0926052	ND	negative	negative
354	0926081	negative	negative	negative
355	0941985	negative	negative	negative
356	0926080	negative	negative	negative
357	0937866	negative	negative	negative
358	0937882	negative	negative	negative
359	0937864	negative	negative	negative
360	0937861	negative	negative	negative

**Example 5****Influence of reaction mix composition on PCR precision**

Goal of the experiment was to analyse whether the reaction mix composition has a significant influence on the precision of the PCR reaction targeting the Parvovirus B19 NS1 region. The primer combination STS17/18 (0.4 µM final concentration each) and the probe STS15 (0.1 µM final concentration) have been added to the reaction mix described in Example 4 as well as to two preformulated reaction mixes which are commercially available (COBAS TaqMan Generic DNA Amplification Kit, COBAS TaqMan Generic RNA Amplification Kit). The Pelispy Parvo-B19-DNA run control at 1E4 IU/mL as described in Example 3 has been extracted in 20 replicates per reaction mix and analysed according to Example 1. Figure 7

5

10

demonstrates that the commercial RNA reaction mix comprises the lowest ct-values along with the best precision as judged by lowest CV of 0.5% compared to the commercial DNA reaction mix with a CV of 4,4% and the inhouse reaction mix with a CV of 2.3% (Table 4).

- 5 A survey of the target region within the genome of parvovirus B19 is shown in Fig. 8.

Table 4:

ct-values with primer combination STS17/STS18		
inhouse reaction mix	commercial generic RNA reaction mix	commercial generic DNA reaction mix
35,2	31,2	47,7
35,5	31,3	47,4
35,2	31,1	49,1
35,8	31,1	49,2
34,9	30,8	50,6
35,3	31,1	48,6
36,0	30,6	44,8
34,8	31,1	51,8
34,7	31,2	50,1
35,2	31,2	47,9
35,1	31,2	49,6
36,3	31,2	48,6
37,3	31,0	52,1
35,5	31,0	50,9
34,9	31,1	50,1
35,7	31,1	49,9
37,3	31,2	55,9
35,9	31,2	52,1
37,2	31,1	48,9
36,6	31,4	49,4
mean	35,7	31,1
SD	0,811	0,166
CV	2,27	0,53

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**Patent Claims**

1. A method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of
  - (a) providing a sample suspected to contain the target nucleic acid
  - 5 (b) providing a pair of primers comprising a first and a second primer whereby the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4,
  - (c) amplifying the target nucleic acid,
  - (d) detecting the amplified target nucleic acid of step (c).
- 15 2. A method according to claim 1 wherein the first primer consists of at least 12 contiguous nucleotides of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 6 or 7 and wherein the second primer consists of at least 12 contiguous nucleotides of the complementary sequence of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 8 or 9.
- 20 3. A method according to claim 1 or 2 wherein the first primer has a nucleic acid sequence selected from the group of nucleic acid sequences SEQ ID NO: 12 to 15 and wherein the second primer has a nucleic acid sequence selected from the complementary sequence of the group of nucleic acid sequences SEQ ID NO: 16 to 17.
- 25 4. A method for the detection of a target nucleic acid comprising the nucleic acid sequence of parvovirus B19 in a sample comprising the steps of
  - (a) providing a sample suspected to contain the target nucleic acid,
  - (b) providing a pair of primers comprising a first and a second primer,
  - (c) amplifying the target nucleic acid,
  - 30 (d) contacting the sample with a probe under conditions for binding the probe to the target nucleic acid,

- 40 -

- (e) detecting the binding product between the target nucleic acid and the probe as an indication of the presence of the target nucleic acid

characterized in that

5 the first primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the nucleic acid sequence SEQ ID NO: 2, and whereby the second primer consists of at least contiguous 12 nucleotides of a nucleic acid sequence selected from the complementary sequence of the nucleic acid sequences SEQ ID NO: 3 or 4, and/or

10 the probe consists of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 5 or a complementary sequence thereof.

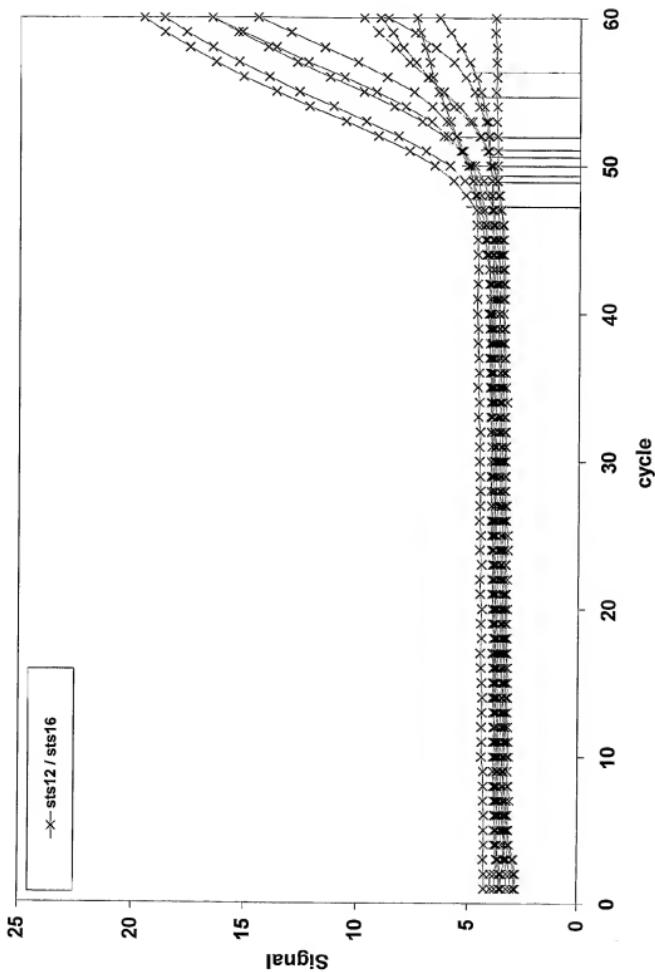
5. The method according to claim 4 wherein the probe carries a label.
6. A method according to claim 5 wherein an additional probe carrying a label is contacted with the sample in step d) so that a pair of probes consisting of a first and a second probe is contacted with the sample in step d).
- 15 7. A method according to claim 6 wherein said amplifying step c) comprises contacting the sample with the said pair of primers to produce an amplification product if the target nucleic acid is present in said sample, wherein said hybridizing step d) comprises contacting said sample with the pair of probes, wherein the members of said pair of probes hybridize to said amplification product within no more than five nucleotides of each other, wherein the first probe of said pair of probes is labeled with a donor fluorescent label and wherein the second probe of said pair of probes is labeled with a corresponding acceptor fluorescent label;
- 20 25 and detecting the binding product between the target nucleic acid and the pair of probes in step e) by detecting the presence or absence of fluorescence resonance energy transfer between said donor fluorescent label of said first probe and said acceptor fluorescent label of said second probe, wherein the presence of fluorescence resonance energy transfer is indicative of the presence of the target nucleic acid in the sample, and wherein the absence of

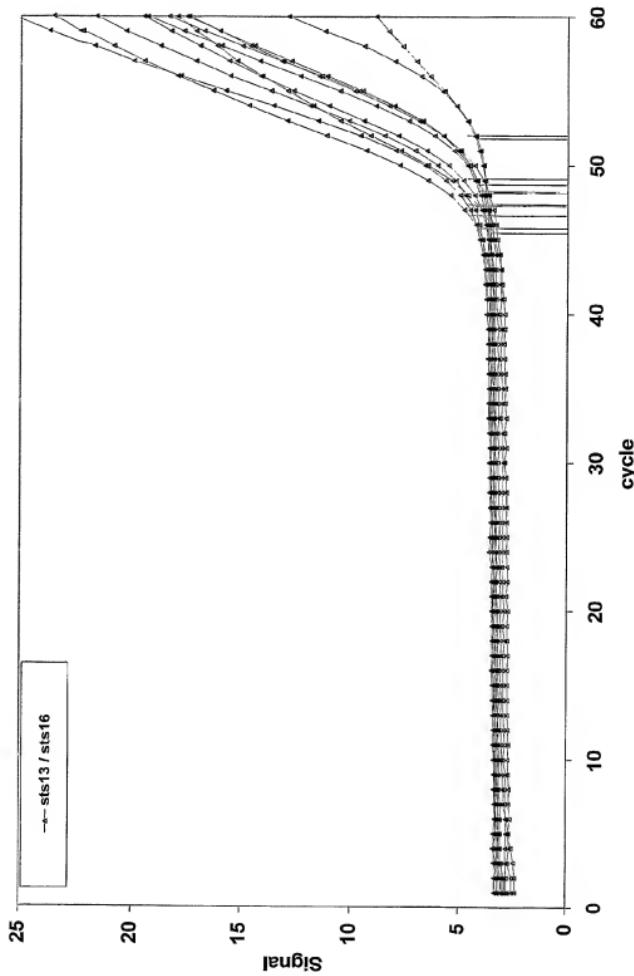
fluorescence resonance energy transfer is indicative of the absence of the target nucleic acid in the sample.

8. The method according to claim 4 wherein the probe carries a first and a second label.
- 5 9. The method according to any of the claims 4 to 8, wherein the target nucleic in step c) is amplified with a template-dependent DNA polymerase.
10. The method according to any of the claims 8 to 9 whereby the binding product between the target nucleic acid and the probe in step (e) is detected by the quantity of the first or second fluorescent label that is released from the probe hybridized to the target nucleic acid by exonuclease hydrolysis by the template-dependent DNA polymerase.
11. A method according to any of the claims 4 to 10 wherein the probe consists of at least 12 contiguous nucleotides of the nucleic acid sequence SEQ ID NO: 10 or a complementary sequence thereof.
- 15 12. A method according to claim any of the claims 4 to 11 wherein the probe has the nucleic acid sequence SEQ ID NO: 11 or a complementary sequence thereof.
13. A method according to any of the claims 4 to 12 wherein the first primer consists of at least 12 contiguous nucleotides of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 6 or 7 and wherein the second primer consists of at least 12 contiguous nucleotides of the complementary sequence of a nucleic acid sequence selected from the nucleic acid sequences SEQ ID NO: 8 or 9.
- 20 14. A method according to any of the claims 4 to 13 wherein the first primer has a nucleic acid sequence selected from the group of nucleic acid sequences SEQ ID NO: 12 to 15 and wherein the second primer has a nucleic acid sequence selected from the complementary sequence of the group of nucleic acid sequences SEQ ID NO: 16 to 17.

15. A method according to any of the claims 4 to 14 wherein the primer and/ or the probe comprise a modified nucleotide or a non-nucleotide compound.
16. A method according to any of the claims 4 to 15 wherein other target nucleic acids are detected in the same reaction.
- 5 17. A method according to claim 16 wherein the other target nucleic acids comprise nucleic acid from hepatitis A virus, hepatitis B virus, hepatitis C virus, west nile virus or the human immunodeficiency virus.
- 10 18. An oligonucleotide whereby the nucleic acid sequence of the oligonucleotide is selected from the nucleic acid sequences SEQ ID NO: 12 to 15, from the nucleic acid sequences 10 or 11 or a complementary sequence thereof or from the complementary sequence of the nucleic acid sequences 16 or 17.
19. An oligonucleotide according to claim 19 which comprises a modified nucleotide or a non-nucleotide compound.
20. A pair of primers comprising a first and a second primer whereby the nucleic acid sequence of the first primer is selected from the nucleic acid sequences SEQ ID NO: 12 to 15 and whereby the nucleic acid sequence of the second primer is selected from the complementary sequence of the nucleic acid sequences 16 or 17.
- 15 21. Use of an oligonucleotide according to any of the claims 18 to 19 or a pair of primers according to claim 20 in a hybridisation reaction with a complementary nucleic acid.
- 20 22. Use of an oligonucleotide according to any of the claims 18 to 19 as a primer, probe or capture probe.
- 25 23. A kit comprising a template dependent DNA polymerase, nucleotides and an oligonucleotide according to any of the claims 18 to 19 or a pair of primers according to claim 20.

1/8

**Fig. 1**

**Fig. 2**

3/8

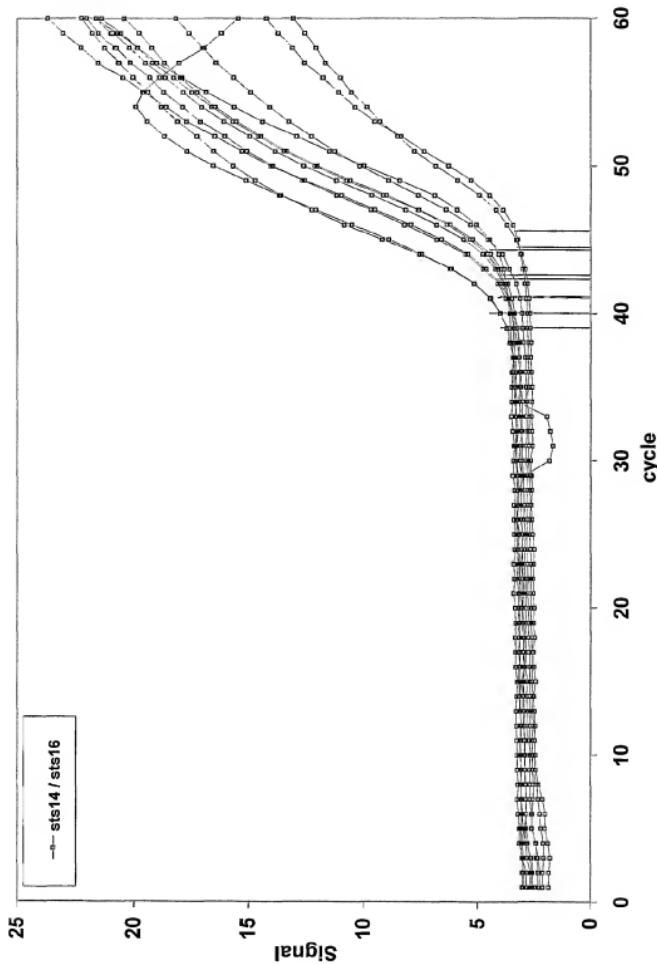
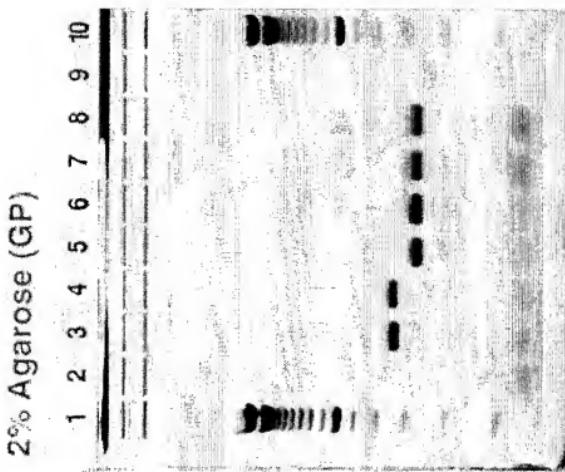
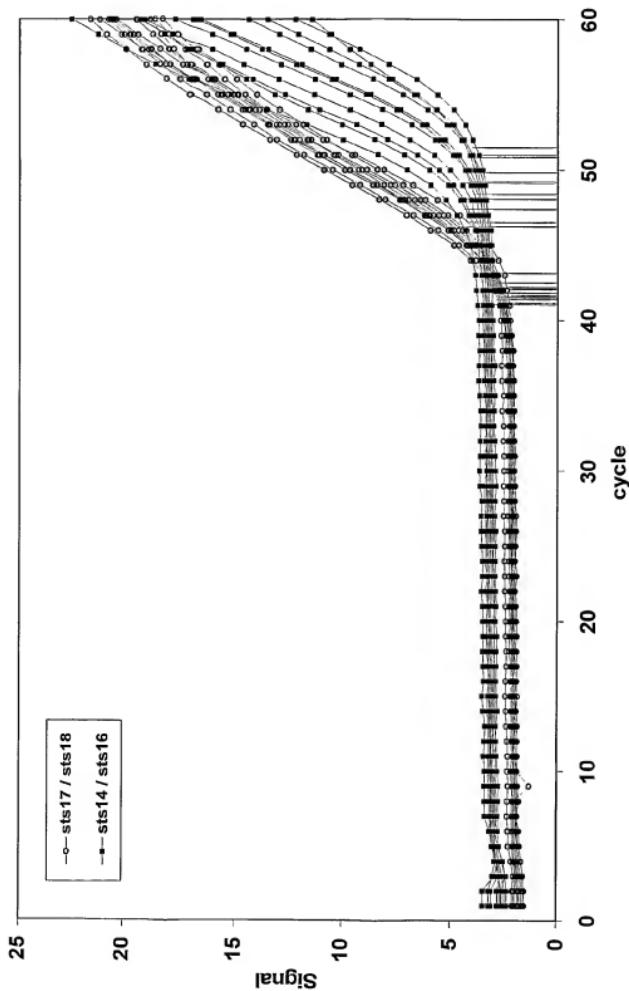
**Fig. 3**

Fig. 4

4/8

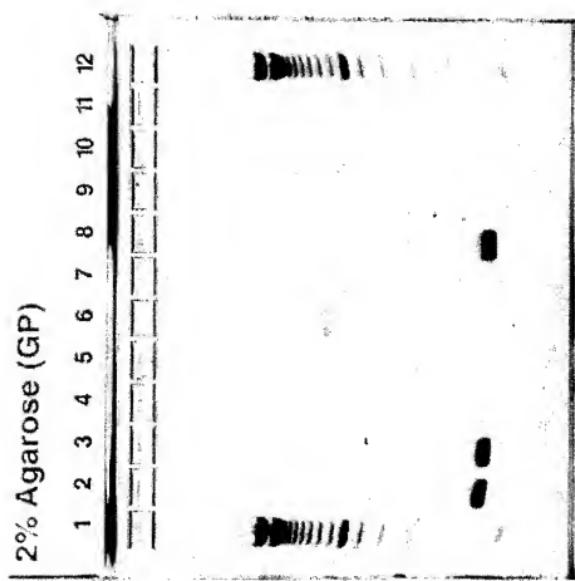


5/8

**Fig. 5**

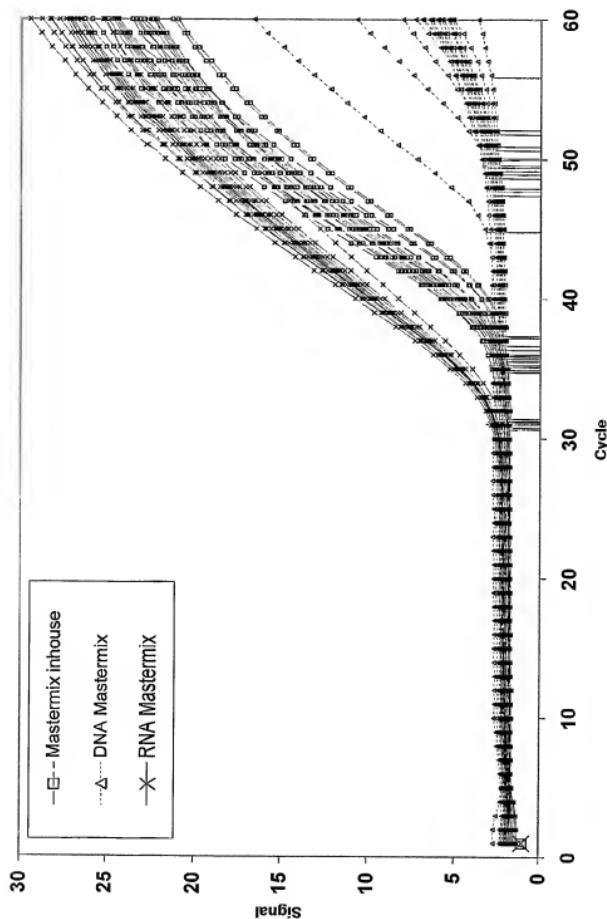
**Fig. 6**

6/8



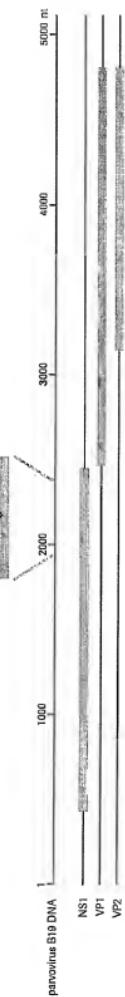
**Fig. 7**

7/8



**Fig. 8:**

8/8



- 1 -

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<151> 2004-02-10

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP2005/001243A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, WPI Data, PAJ, EMBASE, BIOSIS

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X	WO 99/28439 A (ASSIST PUBL HOPITAUX DE PARIS ; AUGUSTE VERONIQUE (FR); GARBARC CHENON) 10 June 1999 (1999-06-10) cited in the application page 17 - page 18; example 4; sequences 11-15, 58, 106, 107, 121	1-5, 9, 11-23
Y	-----	1-23
Y	SCHMIDT I ET AL: "Parvovirus B19 DNA in plasma pools and plasma derivatives." VOX SANGUINIS. NOV 2001, vol. 81, no. 4, November 2001 (2001-11), pages 228-235, XP002288712 ISSN: 0042-9007 the whole document -----	1-23 ----

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search

20 May 2005

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31/05/2005

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Schmitt, A

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP2005/001243

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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